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(19) Europäisches Patentamt

European Patent Office

Office européen des brevets

(11) Publication number:

0 400 920

A1

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 90305731.3

(51) Int. Cl.<sup>5</sup>: C07K 1/04

(22) Date of filing: 25.05.90

(30) Priority: 26.05.89 US 357987

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(43) Date of publication of application:  
05.12.90 Bulletin 90/49

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(84) Designated Contracting States:  
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) Solid phase multiple peptide synthesis.

(57) Methods and compositions are provided, where oligopeptides are produced on a transparent surface while retaining transparency by the cyclical addition of protected monomers. Reagents are specifically selected to allow for efficient reproducible addition, while maintaining transparency of the support. Linkers are provided which permit retention of the oligopeptide to the surface or release of the oligopeptide at completion of the preparation of the oligopeptide. The oligopeptide bound to the support finds use in diagnostic assays, as well as other application, while the free oligopeptides may be used in a variety of ways.

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## SOLID PHASE MULTIPLE PEPTIDE SYNTHESIS

The field of the subject invention concerns peptide synthesis on a solid support.

The investigation of biological processes has greatly expanded with the advent of cloning, sequencing, restriction enzymes, gene banks, improved diagnostic equipment, as well as many other advances which have allowed for the increasing ability to investigate the mechanisms of physiological processes and the molecules involved with such processes. As increasing numbers of biological molecules are isolated and characterized, there has been a concomitant interest in the preparation of peptides. In many physiological processes, oligopeptides play a central role in the process. This is exemplified by the interaction between antigen presenting cells, such as macrophage and B-cells, with T-cells, where the oligopeptide must bind to the MHC antigen and be recognized by the T-cell receptor in conjunction with MHC antigen. Even where the whole protein plays a role, frequently only a small sequence of the protein can mimic a substantial proportion of the activity of the intact protein. Thus, epitopic sites which bind to immunoglobulins may be used for binding to the antibodies, as haptens to induce antibody formation or may be used in diagnostic assays for detecting proteins or their complementary receptors. Peptides may also play a significant role in screening for compounds which may be active as drugs or may provide for insights in the conformation required for agonist or antagonist activity. The preparation of a series of oligopeptides may also serve to define the binding site of receptors and the effect of transduction of signals as a result of changes in sequence.

There is, therefore, substantial interest in methods which provide for simple and efficient techniques for the production of a variety of oligopeptides of different composition.

20 PCT applications WO84/03564, WO84/03506, and WO86/00991 provide techniques for the production of a plurality of peptides. See also the references cited therein. Co-pending application Serial No. 051,917, filed May 19, 1987, describes methods for functionalizing polystyrene surfaces.

25 According to the present invention, functionalized polystyrene surfaces having an active displaceable halide or pseudohalide are substituted with an aminoalkyl or hydroxylalkyl chain as a linker. The resulting linking group is then reacted with an appropriately protected amino acid activated ester, followed by deprotection and extension with additional protected amino acids. Optionally, the linker may be further extended with a benzyl alcohol group. Linkers employing the hydroxyl group are first reacted with a carboxyl functionality more reactive than a pentafluorophenyl activated ester, e.g. symmetric anhydride, where subsequent amino acids are added as the pentafluorophenyl or the like activated ester.

30 Methods and compositions are provided for producing efficiently and rapidly, usually substantially concurrently, a plurality of oligopeptides with a high order of fidelity. The method employs an activated polystyrene surface terminating in an amino or hydroxyl group. The oligopeptide is then prepared by sequentially adding activated carboxyl protected amino acids employing specific reagents for the coupling and deprotection in each cycle.

35 The first stage of the process is the activation of polystyrene. The polystyrene may be present in a variety of forms, such as multiwell plates, rods, sheets, beads, substantially closed or open containers, flasks, where specific areas on the sheet may be dimpled, separated by raised lines or other barriers, or the like. Desirably, the polystyrene is fabricated, e.g. extruded or molded where rods may be chopped to provide beads. The characteristics of the device are that it will allow for the simultaneous manipulation at a plurality of sites of the sequential addition of the same or different amino acids and the same or different reagents for each cycle of addition.

40 The functionalization is extensively described in co-pending application Serial No. 330,207, filed March 29, 1989. In this method, an  $\alpha$ -hydroxylalkyl-2-substituted-acetamide is employed where the substituent is a halide or pseudohalide. For the most part, the polystyrene objects which are activated, will be primarily non-crosslinked, usually having fewer than about 0.5 percent crosslinks, more usually fewer than about 0.1 percent crosslinks. The polystyrene products are normally fabricated, molded, or extruded so as to provide for a smooth surface with few indentations or canyons. With these products, molecules may be bound to the surface and be readily available for binding to other molecules. By employing the subject process with the molded or extruded polystyrene, the polystyrene remains substantially transparent, so that the progress 45 of the reaction may be monitored.

45 Retention of the desirable properties of the fabricated polystyrene is achieved using functionalized Y-methyl acetamides, where the Y-methyl group reacts with the polystyrene in a polystyrene non-swelling or non-dissolving solvent in the presence of a Lewis acid catalyst under mild conditions, and Y is a moiety capable of undergoing nucleophilic substitution by an aryl group, e.g. benzene in the presence of a Lewis acid catalyst. The  $\alpha$ -acyl functional group of the Y-methyl amide is a group which is stable under the

aromatic electrophilic substitution conditions, but can be readily displaced by a Lewis base under nucleophilic substitution conditions.

The compounds used for functionalizing the polystyrene will for the most part have the following formula



wherein:

R is an alkyl group of from 1 to 3, more usually from 1 to 2 carbon atoms or hydrogen, usually hydrogen; X is a halogen (including pseudohalogen), where the halogen is of atomic number of at least 17, wherein X may be chlorine, bromine, or iodine, or a pseudohalogen, such as arylsulfonate esters, sulfonium salts, or the like. Usually, when other than halogen, the substituent on the  $\alpha$ -carbon atom of the acyl group will be of at least about 2 carbon atoms and not more than about 10 carbon atoms, usually not more than about 8 carbon atoms, and may have from 1 to 6, more usually from 1 to 5, and preferably from 1 to 4 heteroatoms, which include halogen and chalcogen (oxygen and sulfur), where any counterion is not included in the limitations. While many other functional groups may be present, such as cyano and nitro, for the most part they do not play a useful role and the compounds are not generally available.

Y may be the same or different from X, usually different, and is a group capable of nucleophilic substitution, particularly oxy-derivatives, halides and pseudohalides, where the oxy-derivatives may be esters, either organic or inorganic, or ethers. Y will usually be of not more than 20 carbon atoms, usually not more than 12 carbon atoms, and up to about 8 heteroatoms, usually not more than about 6 heteroatoms. Y groups 20 include hydroxy, halogen of atomic number of at least 17, pseudohalides such as arylsulfonate esters, esters such as phosphates, carboxylates, imidates, etc, ethers, such as alkyl, aryl, oxyalkyl, etc.

N-hydroxymethyl acetamides which find use are the 2-substituted N-hydroxymethyl acetamides, including but not limited to N-(hydroxymethyl) 2-chloroacetamide, N-(hydroxymethyl) 2-bromoacetamide, N-(hydroxymethyl)-2-iodoacetamide, N-(hydroxymethyl), O-(pbromobenzenesulfonyl) glycolamide, N-(hydroxymethyl), O-(p-toluenesulfonyl) glycolamide, N-(hydroxymethyl)-2-acetamido-pentamethylenesulfonium iodide, and N-(hydroxymethyl) 2-acetamido pentamethylenesulfonium trifluoroacetate. The synthetic preparation for each of these individual compounds is shown in the Examples.

The N-hydroxymethyl compounds may be derivatized or prepared directly with the particular acid or hydroxyl replacing group in the reaction mixture during the N-substitution of the  $\alpha$ -substituted acetamide or by using a formaldehyde derivative such as  $\alpha$ -chloromethyl methoxyethyl ether.

The activation of the polystyrene will occur under mild conditions in a solvent which neither dissolves nor swells the polystyrene. While the preferred solvent is TMS, other solvents which are less efficient may find use, such as acetonitrile, nitromethane, and DMSO (dimethyl sulfoxide).

35 The reaction will be carried out in the presence of a strong acid, particularly a protonic acid, preferably an organic acid, such as trifluoroacetic acid, trifluoromethanesulfonic acid, methanesulfonic acid, sulfuric acid, phosphoric acid, or hydrogen fluoride. The concentration of the acid will generally range from about 0.1 to 2 M. The reaction will normally be carried out in the substantial absence of atmospheric moisture, which can be achieved by covering the reaction mixture, providing for an inert atmosphere, or the like.

40 The polystyrene surface is contacted with the N-(Y-methyl) acetamide compound within a temperature and time range suitable for activating the surface of the substrate. Generally, the temperature will range from about -5 °C to about 60 °C, preferably about 0 °C to about 30 °C, and the activation time is from about 5 min to about 48 h, usually from about 2 to about 8 h. The concentration of the N-(Y-methyl) acetamide compound is typically between about 0.01 to 2 M, usually between about 0.05 to 0.5 M. For most applications, the use of at least about 0.05, usually 0.1 ml of reaction solution per square centimeter of solid substrate surface area is sufficient to provide for the desired level of activation by reaction of electrophilic groups on the surface of the substrate.

45 Before the conjugation of the macromolecule, the surface of the substrate may be freed of any unreacted N-(Y-methyl) acetamide compound by washing with a buffer and/or water. The particular material employed for the washing step is not critical to the invention, and is chosen for convenience, so long as the conditions do not affect the substitutable group of the amide. Substitution of the substitutable group may be conducted immediately after activation, or at some interval of time, since the surface is stable when stored in an inert environment.

50 After activation of the polystyrene surface, the linking group is formed by replacement of the halide or pseudohalide present on the surface. Different groups may be employed for substitution of the halogen or pseudohalogen, including thiol, oxy or amino, preferably thiol. The conditions for the nucleophilic substitution will be substantially conventional, generally employing a non-dissolving, non-swelling solvent media such as water and aqueous buffers, TMS, DMSO, aliphatic alcohols, at a temperature in the range of -5 to

60 °C, usually 0 to 30 °C, usually in the presence of base, usually at a pH in the range of about 8 to 14. The time for the reaction may vary from about 5 min to 12 h. The substituting group will provide for an available amino or hydroxyl group for bonding of the next molecule. The compounds employed will generally be from about 2 to 8 carbon atoms, more usually from 2 to 6 carbon atoms, and preferably from 2 to 4 carbon atoms, more preferably 2 carbon atoms. For the most part, the compounds will be aliphatic and aliphatically saturated. During the reaction, the amino group may be protected or unprotected, depending upon the nature of the substituting group. Protective groups may include benzyloxycarbonyl, acetyl, butyloxycarbonyl, or the like.

Illustrative compounds include aminoethylmercaptan, mercaptoethanol, ethylene diamine, other alkylene diamines and triamines.

In some instances, it may be desirable to further extend the linking group by providing for an acid, base, or hydrazine cleavable benzyl alcohol functionality for preparing the oligopeptide. Various derivatives of benzyl alcohol may be employed for reacting with the available amino group, where the extending molecule bonded to the amino group will have at least 7 carbon atoms and not more than about 14 carbon atoms, usually not more than about 12 carbon atoms. The benzyl alcohol which provides for an ester linkage allows for removal and isolation of the oligopeptide which is produced. In contrast, where it is intended that the oligopeptide remain bonded to the polystyrene solid support, the stabler amide linker will be preferred.

For the most part, the oligopeptides which are produced will be comprised solely of amino acids either the natural carrying L-amino acid or the normally, unnaturally occurring D-amino acid. However, molecules other than amino acids may be included internal to the chain or as the terminal group, as desired. Unnatural amino acids may also be employed, such as p-aminobenzoic acid, 4-aminobutyric acid, or the like. In referring to amino acid, it is intended to use the term generically, in that the molecule has a carboxyl functionality and an amino functionality.

For the purposes of the subject invention, it is desirable that the transparency of the polystyrene be retained. Therefore, a process has been devised which is efficient, provides a high level of fidelity, uses generally available reagents, and can be readily reproduced. In the system, solvents and reagents which would react with or act on the polystyrene are avoided. The amino acid reactants are selected for solubility in the solvents which are employed. The amino acids will usually have the terminal amino group protected, employing conventional protective groups. For the subject invention, the fluorenylmethoxycarbonyl has been found to be effective and is preferred. Other groups may find use as appropriate.

The monomers which are employed will be activated so as to be able to react with the functional group to which they are to be attached. For the most part, carboxyl groups will be involved which will be activated in a variety of ways, particularly as anhydrides or activated esters. The anhydrides will, for the most part, be symmetrical anhydrides, although in some situations, mixed anhydrides may also be employed. Mixed anhydrides will usually involve ester carbonate anhydrides of the monomer. Alternatively, activated esters may be employed, where a variety of alcohols or phenols may be employed. In many instances, different esterifying agents will be employed, depending upon the particular monomer. Esterifying agents of particular interest include pentafluorophenol, 1-hydroxybenzotriazole, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, 4-nitrophenol, 2,4-dinitrophenol, N-hydroxysuccinimide, and pentachlorophenol.

The activated monomer will be added to the linking group or growing chain in a non-hazing solvent. Desirably, a combination of tetramethylene sulfone and dimethyl sulfoxide will be employed as the solvent, generally in the ratio of about 1:3-3:1, preferably about 1:1. The time for the reaction will vary, generally ranging from about at least 0.5h and not more than about 3h, generally ranging from about 1 to 2h. The concentration of the monomer will generally be in the range of about 0.05 to 1.0M. Desirably, room temperature is employed, although lower or higher temperatures may be employed, generally in the range of about 4-60 °C.

After completion of the reaction, the solution may be removed and any unreacted amino groups capped. For capping, acetylating agents may be employed, conveniently 1-acetylimidazole. A solution of the capping agent in 1:1 TMS/DMSO will be employed for at least about 0.5h and generally not exceeding about 3h, usually not exceeding about 2h, 1h generally being satisfactory. The concentration of the capping agent will usually be at least about 0.5M, and not more than about 1.5M, more usually not more than about 1M.

After completion of capping, the protective groups will be removed. Generally, heterocyclic amines will be employed, where the nitrogen is a member of the ring, particularly rings of from 5 to 6 carbon atoms and desirably having an hydroxyl group. Preferred is 4-hydroxypiperidine. The concentration of the deprotecting amine will generally be in the range of about 0.1 to 1.0M and deprotection will generally be carried out at about room temperature, although any temperature from 10 ° to 30 °C may be employed. The

solvent will be the same solvent as used previously.

The deprotecting solution is then removed and the cycle repeated by adding the next monomer. The process will usually be carried out for at least 5 cycles and may be carried out for 25 cycles or more. It is understood that the yield will diminish with each cycle, although the subject method provides for high yields with peptides not exceeding 15 monomers.

At the completion of the synthesis, the terminal amino group will be deprotected as described above, followed by deprotection of the side chain functionalities. Since the side chain functionalities will vary depending upon the particular monomer, deprotection conditions will be employed which are sufficient for the side group protecting functionality requiring the most rigorous conditions. Where less rigorous conditions suffice, the less rigorous conditions may be employed. For the most part, it has been found that various acidic treatments may be employed, conveniently 95% trifluoroacetic acid in water for at least about 120 min., preferably at least 180 min. at room temperature. Where the peptide is linked to the solid support by an ester link, the acid hydrolysis also releases the peptide from the support.

The subject invention provides minimum purities of at least about 70% for oligopeptides in excess of about 10 amino acids. In addition, per well of a multiwell plate, one nanomole or more may be obtained.

The peptides as bound to the support may find a wide variety of uses. Conveniently, the peptides may be prepared as ligands for use in diagnostic assays. Thus, the peptide will be cross reactive with a ligand of interest, so that enzyme-antibody conjugates may bind to the oligopeptide on the support in competition with analyte in the medium. Conventional assays, such ELISA assays may be employed with advantage. See, for example, U.S. Patent No. 3,850,752. Thus, plates may be pre-prepared which may be used directly in ELISA assays for ligands of interest.

In addition, the oligopeptide containing plates may be used for screening receptors, isolating cells, receptors, antibodies or the like, which specifically bind to the oligopeptides, investigating structural conformation of such binding molecules by virtue of varying the oligopeptide sequence, and the like.

The following examples are offered by way of illustration and not by way of limitation.

## EXPERIMENTAL

30

Dynatech/polystyrene microtiter plates were functionalized with functionalized with N-hydroxymethyl 2-bromoacetamide (BA) as follows. A 0.2M solution of N-hydroxymethyl 2-bromoacetamide in tetramethylenesulfone (TMS) was combined in a 1:1 ratio with a 2M trifluoromethylsulfonic acid (TMSA) and the reaction solution was immediately added to the microtiter plate wells. The reaction solution was left in contact with the polystyrene surface for 5h at room temperature, washed with water until odorless, and air dried at room temperature overnight. The surfaces were stable for at least two weeks in a dry, light free environment. At least 0.1ml of reaction solution per square centimeter of polystyrene surface was used.

### 40 POLYSTYRENE ACTIVATION (AEA):

The BA activated surface was reacted with 2-aminoethanethiol (Aldrich) to provide the Aminoethylacetamide (AEA) primary amine surface for peptide synthesis, using a 0.15M solution in 1:1 v/v TMS/DMSO or 0.25M Na<sub>2</sub>CO<sub>3</sub> in H<sub>2</sub>O.

45 The conversion of the BA surface to the AEA surface was tested by treating AEA wells with 5 mM bromophenol blue (BPB) in DMSO. The dye forms an ion pair with amine groups. The dye can be released using 50 mM carbonate pH = 9.2 containing 10% DMSO. The soluble dye absorbance at 570nm (subtracting background at 650nm) is measured with an ELISA plate reader. This technique was employed frequently to qualitatively monitor the coupling and deprotection steps of solid phase multiple peptide synthesis (SPMPS).

50 The data in Table 1 demonstrates the application of BPB/amine binding to study the conversion of the BA surface to the AEA surface. The transformation was also validated by ESCA. There was a low level of non-specific dye staining for the polystyrene and BA surfaces.

TABLE 1

BROMOPHENOL BLUE DYE BINDING TO AN AEA PLATE		
	Polystyrene	AEA Surface
5	0.002±.001	0.004±.001
10		0.767±.084

PEPTIDE SYNTHESIS (Stability):

15 The stability of the peptide synthesis surface to methyl sulfoxide (DMSO) and a 1:1 mixture of DMSO and tetramethylene sulfone (TMS) was tested. The Fmoc-amino acid-OPfp esters were not completely soluble in TMS alone, but quite soluble in DMSO. (Fmoc = fluorenylmethoxycarbonyl, OPfp = pentafluorophenyl) A 1:1 mixture of TMS:DMSO was employed as solvent. The solvent system was compatible with automated pipetter systems, (i.e., nondestructive to the hardware).

20 A stability study was performed coupling alanine to the SPMPS surface and carrying out 15 cycles of peptide synthesis, excluding the use of Fmoc-amino acid-OPfp esters (i.e., just solvent) due to the expense of the reagents. Because analysis for alanine was required at the end of 5, 10, and 15 cycles, the acid cleavable linker 4-hydroxymethylphenoxyacetic acid was coupled to the AEA plate via its pentafluorophenyl ester (see below) and Fmoc-alanine symmetric anhydride was coupled to the linker plate by conventional 25 methods using 0.1M diisopropyl carbodiimide, 0.1M Fmoc-alanine, 0.05M dimethylamino-pyridine, and the reaction allowed to proceed for 90 min. The synthesis cycles were then performed by the Beckman Biomek 1000 automated pipetter.

30 After 5, 10 and 15 cycles the wells were treated with TFA/H<sub>2</sub>O (95:5) to release the alanine, which was quantitated by amino acid analysis. The AEA + linker + alanine is stable to TMS/DMSO for at least 15 cycles. DMSO alone caused a decrease in recovered alanine by 67% over 15 cycles.

35 After cleaving the alanine from the peptide synthesis surface, water was added to the wells and the plate was read in an ELISA reader at 570nm. The absorbance data at 0 (polystyrene), 5, 10, and 15 cycles of SPMPS are shown in Table 2. The peptide synthesis surface showed no increase in optical density. If hazing had occurred the optical density would have increased with the number of SPMPS cycles.

TABLE 2

HAZING OF THE SPMPS SURFACE DETERMINED BY OPTICAL DENSITY	
SPMPS Cycles	A570
40	0 CYCLES 0.046
45	5 CYCLES 0.046
50	10 CYCLES 0.047
	15 CYCLES 0.047

PEPTIDE SYNTHESIS (Capping):

55 When it was necessary to cap uncoupled free amino groups, 1-acetylimidazole was used. Capping was performed with 0.5M acetylimidazole in 1:1 TMS/DMSO for 1 h at room temperature.

The decrease in BPB binding was used to indicate capping efficiency. Since the capping reagent is

sensitive to water, reaction time was kept to 1.0 hour at room temperature. From this data, a capping concentration of 0.8M was selected with a reaction time of 1.0 hour.

5 PEPTIDE SYNTHESIS (Deprotection):

The time dependence of the deprotection step was studied using an AEA plate coupled with Fmoc-glycine. The deprotection of the Fmoc group by 4-hydroxypiperidine was monitored over time using the bromophenol blue test at regular intervals. Maximum dye binding (and thus maximum deprotection of the amine group) was reached at approximately 6 minutes. For SPMPS, deprotection was allowed to go for 20 minutes to insure a complete deprotection step.

15 PEPTIDE SYNTHESIS (Coupling):

The time dependence of the coupling reaction to an AEA plate with several Fmoc-amino acid pentafluorophenyl esters was studied. Coupling was performed with 0.1M Fmoc-amino acid - OPfp in 1:1 TMS/DMSO for 90 min. In cases of double coupling, after aspiration of the reaction medium, the step is repeated. (see Table 3). The coupling completeness was measured using the bromophenol blue test: a low BPB reading being indicative of high coupling efficiency. After the coupling reaction, the wells were capped and tested with BPB again. To gain insight into the efficiency of the process, the coupled amino acid was deprotected and tested with dye for comparison with the original AEA plate. The comparison only serves as a guide to efficiency, which was quite good for all couplings investigated.

25

TABLE 3

BPB BINDING TO ASSESS COUPLING EFFICIENCY TO AEA SURFACE					
	AMINO ACID COUPLED	AEA WELL	COUPLED FMOC-AA	CAPPED FREE AMINE	DEPROTECTED FMOC-AA
30	FMOC-ARG(PMC)Obt* 90 MINUTES	.427	.017	.008	.414
	FMOC-ARG(PMC)Obt* 180 MINUTES	.368	.013	.008	.451
35	FMOC-PRO-OPfp 90 MINUTES	.425	.091	.017	.325
	FMOC-PRO-OPfp 180 MINUTES	.477	.041	.012	.446
40	FMOC-LYS(BOC)-OPfp 90 MINUTES	.474	.024	.012	.512
	FMOC-LYS(BOC)-OPfp 180 MINUTES	.520	.015	.011	.602
	FMOC-PHE-OPfp 90 MINUTES	.490	.035	.016	.513
	FMOC-PHE-OPfp 180 MINUTES	.549	.019	.013	.607
	FMOC-TRP-OPfp 90 MINUTES	.496	.053	.017	.485
	FMOC-TRP-OPfp 180 MINUTES	.497	.038	.013	.529

\* Obt is 1-hydroxybenzotriazole active ester

45

PEPTIDE SYNTHESIS (Coupling) cont.:

50 Several Fmoc-amino acid active esters were tested for their ability to undergo a relatively unhindered coupling to a glycine residue coupled to AEA. The data in Table 4 reveal that coupling (monitored by reduction in BPB absorbance) is complete after the first coupling of 90 minutes, with a small improvement after the second coupling. The Fmoc-protected dipeptides were deprotected with 4-hydroxypiperidine and tested with BPB for to the starting AEA wells. The qualitative results gleaned by comparing the AEA starting wells to the deprotected Fmoc-AA-(amino-acid) wells indicated a high overall efficiency for coupling two residues. In fact, BPB binding is actually higher for the dipeptides than the starting AEA wells.

TABLE 4

BPB BINDING TO ASSESS COUPLING EFFICIENCY TO GLYCINE COUPLED AEA				
	AMINO ACID COUPLED	COUPLED FMOC-AA-GLY	DEPROTECTED DIPEPTIDE	STARTING AEA WELL
5	FMOC-LYS(BOC)-OPfp	1X .005	.484	.399
	FMOC-LYS(BOC)-OPfp	2X .005	.471	.386
10	FMOC-PHE-OPfp	1X .008	.406	.383
	FMOC-PHE-OPfp	2X .007	.412	.398
15	FMOC-PRO-OPfp	1X .010	.415	.384
	FMOC-PRO-OPfp	2X .008	.432	.393
20	FMOC-SER*-ODhbt**	1X .004	.463	.406
	FMOC-SER*-ODhbt**	2X .004	.458	.403
25	FMOC-TYR*-OPfp	1X .008	.500	.432
	FMOC-TYR*-OPfp	2X .006	.531	.471

\* Side-chain protected as t-butyl ether

\*\* ODhbt is 3,4-dihydro-3-hydroxy-4-oxo-1, 2, 3-benzotriazine active ester.

PEPTIDE SYNTHESIS (Coupling) cont.:

The same group of Fmoc-amino acid-active esters used in the glycine coupling study (Table 5) were tested for their coupling with a more hindered residue: isoleucine previously coupled to AEA. The results in Table 5 point to the necessity for two coupling cycles with more hindered condensations. This is evidenced by the difference between the first and second couplings of the Phe, Pro, and Tyr residues. Again, qualitative comparison of the BPB-dipeptide binding with the starting AEA absorbance would seem to indicate very efficient overall coupling, even with these very hindered coupling reactions.

TABLE 5

BPB BINDING TO ASSESS COUPLING EFFICIENCY TO ISOLEUCINE COUPLED AEA				
	AMINO ACID COUPLED	COUPLED FMOC-AA-ILE	DEPROTECTED DIPEPTIDE	STARTING AEA WELL
35	FMOC-LYS(BOC)-OPfp	1X .006	.427	.402
	FMOC-LYS(BOC)-OPfp	2X .005	.409	.395
40	FMOC-PHE-OPfp	1X .012	.331	.383
	FMOC-PHE-OPfp	2X .006	.338	.381
45	FMOC-PRO-OPfp	1X .015	.330	.379
	FMOC-PRO-OPfp	2X .006	.352	.399
50	FMOC-SER*-ODhbt**	1X .004	.422	.400
	FMOC-SER*-ODhbt**	2X .003	.442	.423
55	FMOC-TYR*-OPfp	1X .015	.396	.426
	FMOC-TYR*-OPfp	2X .006	.523	.498

\* Side-chain protected as t-butyl ether

\*\* ODhbt is 3,4-dihydro-3-hydroxy-4-oxo-1, 2, 3-benzotriazine active ester.

In both the direct and linker SPMPS methods, the Fmoc group of the N-terminal residue is removed before side-chain deprotection. The side-chain protecting groups (t-butyl esters, t-butyl ethers, butoxycarbonyl (Boc) and 2,2,5,7,8-(pentamethylchroman-6-sulphonyl) (PMC) are then cleaved by acid treatment.

A time course study of side-chain deprotection is shown in Table 6. Different acid/water combinations were studied for their ability to effect the deprotection of Lys(Boc) and Arg(PMC) side chain protecting groups, since these side-chains were most relevant to the synthesis of  $\alpha$ -MSH.

TABLE 6

BPB BINDING TO DEPROTECTED BASIC RESIDUES WITH TIME							
	AMINO ACID	T = 0	T = 5	T = 15	T = 30	T = 60	T = 90
FMOC-LYS(BOC) 90% TFA/WATER	.022	1.146	1.207	1.361	0.953	---	---
FMOC-ARG(PMC) 90% TFA/WATER	.022	0.040	0.064	0.071	0.128	---	---
FMOC-ARG(PMC) 95% TFA/WATER	.022	---	---	---	---	0.146	0.286
FMOC-ARG(PMC) 95% TFA/WATER + 0.5M TFMSA	.022	---	---	---	---	---	0.282
FMOC-ARG(PMC) 95% TFA/WATER + 1.0M TFMSA	0.22	---	---	---	---	---	0.306

20

#### PEPTIDE SYNTHESIS (Side-Chain Deprotection):

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The Boc protecting group reaches the maximum dye binding within the first 30 minutes of the reaction with 95% TFA/H<sub>2</sub>O. The PMC group does not reach a maximum until 180 minutes reaction with 95% TFA/H<sub>2</sub>O. The conclusion from this study was that 95:5 TFA:H<sub>2</sub>O for 3h at room temperature was sufficient to remove very acid labile groups like Boc and t-butyl esters/ethers, as well as less susceptible groups like PMC.

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In direct SPMPS the peptide side chain groups were deprotected with TFA/H<sub>2</sub>O while the peptide remained covalently bonded to the plate. At this point, the peptide was ready to be analyzed by a method such as an ELISA.

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In linker SPMPS (linker is the hydroxymethylphenoxyacetic acids, the peptide side-chain groups were deprotected and the peptide simultaneously cleaved from the plate by TFA/H<sub>2</sub>O. A sequence analysis could be performed directly on the peptide. In addition, amino acid analysis could be performed after hydrolyzing the deprotected peptide with 6N HCl at 110 °C for 24 hours.

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#### AMINO ACID ANALYSIS ( $\alpha$ -MSH):

Amino acid analysis was done on alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) after synthesis using the linker methodology. The data is shown below in Table 7.

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TABLE 7

AMINO ACID ANALYSIS DATA FOR $\alpha$ -MSH				
	AA	THEORY	$\alpha$ -MSH #1	$\alpha$ -MSH #2
10	Val = V	1.000	0.860 ( 9138)	0.878 (4725)
	Pro = P	1.000	1.000 (10627)	1.000 (5381)
	Lys = K	1.000	0.606 ( 6436)	0.531 (2857)
	Gly = G	1.000	0.380 ( 4035)	0.377 (2027)
	Trp = W	1.000	— —	— —
	Arg = R	1.000	0.251 ( 2667)	0.239 (1288)
	Phe = F	1.000	0.236 ( 2794)	0.231 (1244)
	His = H	1.000	0.296 ( 3148)	0.257 (1389)
	Glu = E	1.000	0.264 ( 2806)	0.230 (1240)
	Met = M	1.000	0.100 ( 1048)	0.103 ( 555)
15	Ser = S	2.000	0.555 ( 5900)	0.632 (3400)
	Tyr = Y	1.000	0.176 ( 1872)	0.186 (1000)
( ) = Peak area from amino acid analysis				
Note:				
$\alpha$ -MSH #1 = $\alpha$ -MSH synthesized with capping of the first 7 residues.				
$\alpha$ -MSH #2 and #3 = $\alpha$ -MSH synthesized without.				

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The data indicates no significant difference between the capped and the non-capped syntheses. Analysis for Trp is not possible due to degradation in the 6M HCl acid hydrolysis. Methionine was low, probably, because of oxidation during acid hydrolysis.

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The conclusions from this study are that the proper residues are present in amounts indicating a successful synthesis. This was confirmed by the sequence analysis of  $\alpha$ -MSH (see below). The early residues are in higher amounts than the later residues (a 40% decrease in detected lysine and a further 33% decrease in detected glycine was noted in all three syntheses).

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#### AMINO ACID ANALYSIS (Leu-5-Enkephalin):

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The amino acid analysis of the peptide Leu-5-enkephalin is shown below in Table 8. The ratios for leucine, tyrosine and phenylalanine are near theory. However, the ratio for glycine was found to be significantly higher than theory.

TABLE 8

SEQUENCY ANALYSIS OF LEU-5-ENKEPHALIN			
Amino Acid	Theo. Value	Raw Data	Leu-Enk
Leu = L	1.000	3143	0.643
Phe = F	1.000	4891	1.000
Gly = G	2.000	155488	3.167
Tyr = Y	1.000	4585	0.937

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In the Leu-5-enkephalin work, the possible steric requirement for some of the peptide chains is avoided. This results in much more satisfactory amino acid analysis values. Glycine is 50% higher than theory, which is the result of double couplings occurring or contamination. Double coupling occurs if residual 4-

hydroxypiperidine is present. This is avoided by providing extra washes to the Biomek deprotection subroutine.

5 SEQUENCE ANALYSIS ( $\alpha$ -MSH):

In addition to the amino acid analysis data for  $\alpha$ -MSH #1 (Table 7), sequence analysis was performed, and the data is shown in Table 10. The sequence analysis by repetitive Edman degradation on an Applied Biosystems 420A Derivatizer gave the correct phenylthiohydantoin (PTH) amino acid for all residues in  $\alpha$ -MSH (with the exception of Trp) in greater than 70% yield of the theoretical. Quantitation of the data showed that each well contains approximately 1 nanomole of completed peptide, based on the amount of N-terminal serine detected. An amount of peptide in the picomole range is considered necessary for ELISA detection, so clearly the subject invention provides adequate amounts of peptide synthesized in each well.

In nearly all cases, the main PTH amino acid was detected along with a small percentage of the PTH amino acid corresponding to the next residue in the sequence. This "foreshadowing" is most likely a machine artifact, or a failure to completely couple the last serine residue in  $\alpha$ -MSH. In either case, it suggests that 70% is the minimum purity of the synthesized peptide.

Tryptophan was not detected, although it theoretically should have been present. The primary residue detected where tryptophan should have been found (cycle 9) was glycine, the "foreshadow" residue of tryptophan. This implies that Trp is destroyed by the global deprotection conditions, emphasizing the strong need to develop global deprotection conditions which include the addition of scavengers.

TABLE 9

SEQUENCE ANALYSIS DATA FOR $\alpha$ -MSH					
	Edman Cycle	Theor. PTH Amino Acid	Detected PTH Amino Acid(s)	Yield Picomoles*	% Correct Sequence**
30	1	Ser = S	S (G) (Y)	3147 (613) (470)	74
	2	Tyr = Y	Y (L)	4206 (560)	88
	3	Ser = S	S (M)	3005 (219)	93
	4	Met = M	M (E)	5458 (190)	97
	5	Glu = E	E	2733	100
	6	His = H	H (F)	753 (280)	73
	7	Phe = F	F (R)	2170 (610)	78
	8	Arg = R	R (G)	1465 (340)	81
	9	Trp = W	G (K)	1022 (240)	
	10	Gly = G	G (K)	1703 (690)	71
	11	Lys = K	K (P)	1258 (262)	83
	12	Pro = P	P (V)	393 (90)	81
	13	Val = V	V	145	100

\* Data is based on 8 synthesis wells and 1/3 dilution of the sample. Thus the amount of peptide can be determined by the following: last synthesized residue (serine) equals 3,147 picomoles multiplied by 3/8 yields approximately 1 nanomole per well.

\*\* All PTH amino acids with 3% or greater of the total absorbance for all peaks detected are listed. All other peaks were considered to be background.

50 The ELISA (Enzyme Linked ImmunoSorbent Assay) represents one of the most useful potential applications of the SPMPS technique. An ELISA was accomplished in SPMPS by direct synthesis of the peptide on the AEA surface, followed by acid deprotection of the side-chain protecting groups. The wells were then blocked with bovine serum albumin to reduce non-specific binding. Rabbit anti-( $\alpha$ -MSH) or rabbit anti-Leu-5-enkephalin was added to the wells, and antibodies recognizing the peptide were bound. 55 Detection of the bound rabbit antibodies binding was accomplished by incubation with alkaline phosphatase (AP) conjugated to goat anti-rabbit IgG, followed by washing to remove excess conjugate, and addition of p-nitrophenyl phosphate, a substrate of AP. The AP turned over the substrate to generate p-nitrophenolate, which was spectrophotometrically detected at 405nm in an ELISA plate reader. Dilutions of rabbit IgG

passively adsorbed to a polystyrene microtiter plate were detected with different dilutions of the conjugate. A final dilution of 1:3500 was found to be optimum. Control wells (no rabbit IgG) showed low background binding (absorbance at 405nm-650nm <0.040). This validated the use of this conjugate for the SPMPS ELISA studies.

- 5 An ELISA was run on the pentapeptide (Y-G-G-F-L) Leu-5-enkephalin peptide. It was compared to several other surfaces for binding to rabbit anti-(Leu-5-enkephalin): N-terminal Fmoc enkephalin (FMOC-ENK), Liner-synthesized Leu-5-enkephalin after acid release of peptide (L-ENK), a tripeptide Phe-Gly-Tyr (F-G-Y), and N-terminal acetylated Leu-5-enkephalin (ENK-AC). The data below showed that the strongest immunoreactivity was seen with ENK and ENK-AC, as expected. There was some cross-reactivity with the
- 10 tripeptide F-G-Y. No immunoreactivity was seen for the FMOC-ENK peptide. The slight immunoreactivity observed for the L-ENK wells may indicate that some peptide remains bound to the wells after the acid deprotection.

The following is a flow chart of the process:

	Operation	Reactant	Solvent	Cycles	Time Hours
15 20 25	WASH*	SPMPS SURFACE	TMS/DMSO	3	-----
	Couple	Fmoc-AMINO	TMS/DMSO	-	1.5
	Recouple	Fmoc-AMINO ACID PFP ESTER	TMS/DMSO	-	1.5
	WASH	SPMPS SURFACE	TMS/DMSO	3	-----
	CAPPING**	N-ACETYLIMIDAZOLE	TMS/DMSO	-	1.0
	WASH	SPMPS SURFACE	TMS/DMSO	3	-----
	DEPROTECTION	4-HYDROXYPiperidine	TMS/DMSO	2	-----
	WASH	SPMPS SURFACE	TMS/DMSO	2	-----

\* Start at first wash for all cycles

\*\* Capping was done for "hard" to couple sequences only; BPB test was done after recouple, after capping and/or after deprotection

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It is evident from the above results that the subject methodology provides for a convenient rapid method for producing relatively large amounts of oligopeptides with high fidelity. Thus, devices can be provided, where a surface has a specific sequence or a plurality of different, either related or unrelated sequences, for use in assays, research, and the like. In addition, oligopeptides may be prepared which are easily freed from this support and may find use as drugs, as ligands in competitive assays, and the like. The method allows for optically transparent solid supports, so that the course of the reaction may be observed and where problems occur, the reaction may be stopped. Furthermore, where assays are being carried out, the transparent support allows for light transmission, so that spectrophotometers may be used effectively. Thus, the methodology permits carrying out of fluorescent or enzyme assays, where the label requires detection of light.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

45 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto.

## 50 Claims

1. A method for preparing oligopeptides by means of sequential addition of amino acid monomers bound to an optically clear polystyrene support, said method comprising:
  - (a) reacting a protected activated amino acid monomer with an amino or hydroxyl functionality bonded through a linking group to said polystyrene surface in a reaction solvent comprising a mixture of tetramethylene sulfone and dimethyl sulfoxide to form a bond between said activated ester group and said functionality to provide bound first monomer;
  - (b) removing unreacted amino acid monomer and deprotecting said bound first monomer with a

- cyclic amino group in said reaction solvent to provide available amino acid amino groups;
- (c) reacting said amino acid amino groups with the next protected activated amino acid monomer in said reaction solvent to extend the peptide chain by one additional amino acid;
  - (d) capping any unreacted available amino groups;
  - 5 (e) repeating steps in the order (c), (d) and (b) until the penultimate amino acid monomer is reacted; and
  - (f) repeating steps (c) and (b) with the ultimate amino acid monomer.
- 10 2. A method according to Claim 1, wherein said linking group comprises a methyleneaminoacetyl group.
- 15 3. A method according to Claim 2, wherein said linking group comprises a thioalkyleneamine or hydroxy group bonded through the thio to said acetyl group.
- 20 4. A method according to Claim 1, wherein said polystyrene surface is functionalized by reacting said surface with a compound of the formula:
- $$X(R)CHCONHCH_2Y$$
- 25 wherein:
- R is an alkyl group of from 1 to 3 carbon atoms or hydrogen;
- X is halogen or pseudohalogen; and
- Y is a group capable of nucleophilic substitution;
- in the presence of a strong protonic acid;
- 30 at a temperature in the range of about -5° to 60° C for a time sufficient for said compound to react with said polystyrene at its surface.
- 35 5. A method for preparing oligopeptides by means of sequential addition of amino acid monomers bound to an optically clear polystyrene support, said method comprising:
- (a) reacting a protected activated amino acid monomer with an amino or hydroxyl functionality bonded through a linking group to said polystyrene surface in a reaction solvent comprising a mixture of tetramethylene sulfone and dimethyl sulfoxide to form a bond between said activated ester group and said functionality to provide bound first monomer, wherein said activated amino acid is an ester of pentafluorophenol, 1-hydroxybenzotriazole, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; p-nitrophenol, or N-hydroxysuccinimide;
  - (b) removing unreacted amino acid monomer and deprotecting said bound first monomer with 4-hydroxypiperidine in said reaction solvent to provide available amino acid amino groups;
  - (c) reacting said amino acid amino groups with the next protected activated amino acid monomer in said reaction solvent to extend the peptide chain by one additional amino acid;
  - (d) capping any unreacted available amino groups with 1-acetylimidazole;
  - 40 (e) repeating steps in the order (c), (d) and (b) until the penultimate amino acid monomer is reacted; and
  - (f) repeating steps (c) and (b) with the ultimate amino acid monomer.
- 45 6. A method according to Claim 5, wherein said reaction solvent is a substantially equal mixture of tetramethylene sulfone and dimethyl sulfoxide.
- 50 7. A method according to Claim 6, wherein said linking group terminates in an aliphatic primary amine, aliphatic primary hydroxyl, or a benzyl alcohol.
8. A fabricated polystyrene article characterized by:
- being optically clear;
  - functionalized at the surface at high density with linking groups terminating in an amino or oxy group;
  - 45 linked to said terminating amino or oxy group an amino acid or oligopeptide, wherein said oligopeptide is substantially uniform in composition.
9. An article according to claim 8, wherein said linking group comprises a methyleneaminoacetyl group.
10. An article according to claim 9, wherein said linking group comprises a thioalkyleneamine group bonded through the thio to said acetyl group.
- 55 11. Beads characterized by:
- being optically clear;
  - functionalized at the surface at high density with linking groups terminating in an amino or oxy group;
  - linked to said terminating amino or oxy group an amino acid or oligopeptide, wherein said oligopeptide is substantially uniform in composition.



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## EUROPEAN SEARCH REPORT

Application Number

EP 90 30 5731

DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)
Category	Citation of document with indication, where appropriate, of relevant passages		
A	CHEMICAL ABSTRACTS, vol. 98, no. 13, 28th March 1983, pages 643-644, abstract no. 107756b, Columbus, Ohio, US; S.H. NAKAGAWA et al.: "Synthesis of protected peptide segments and their assembly on a polymer-bound oxime: application to the synthesis of a peptide model for plasma apolipoprotein A-I", & J. ORG. CHEM. 1983, 48(5), 678-85 * Abstract * ---		C 07 K 1/04
A	CHEMICAL ABSTRACTS, vol. 110, no. 11, 13th March 1989, page 761, abstract no. 95779n, Columbus, Ohio, US; M. MERGLER et al.: "Peptide synthesis by a combination of solid-phase and solution methods. I. A new very acid-labile anchor group for the solid phase synthesis of fully protected fragments", & TETRAHEDRON LETT. 1988, 29(32), 4005-8 * Abstract * ---		
A	EP-A-0 273 895 (MONSANTO) ---	1	C 07 K
L	TETRAHEDRON LETTERS, vol. 31, no. 3, 1990, pages 339-340, Pergamon Press Plc, GB; M.S. SHEKHANI et al.: "Carboxamidomethyl ester as useful handle in polystyrene-based peptide synthesis: cleavage of peptide with mercaptide" * Reference 6 * -----	1	
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	02-09-1990	MASTURZO P.	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... & : member of the same patent family, corresponding document	
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